(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number: **0 428 486 A1**

(12) EUROPEAN PATENT APPLICATION

(21) Application number: 90810872.3

(22) Date of filing: 13.11.90

(51) Int. Cl.^{5}: A61K 47/48

(30) Priority: 15.11.89 US 437487

(43) Date of publication of application: 22.05.91 Bulletin 91/21

(84) Designated Contracting States: AT BE CH DE DK ES FR GB GR IT LI LU NL SE

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(54) Polymyxin conjugates.

(57) Water-soluble polymyxin-carrier conjugates such as polymyxin B-dextran conjugates are disclosed. They have a greater half-life in the bloodstream and are significantly more potent and less toxic than native polymyxins.

EP 0 428 486 A1

POLYMYXIN CONJUGATES

The invention relates to novel polymyxin conjugates and to methods of preparing and using them.

Endotoxins or lipopolysaccharides are structural molecules derived from the cell walls of the Gram-negative bacteria. When introduced into the bloodstream, they can interfere with the regulation of body temperature and cause fever. They also have a toxic effect, leading to cardiac, pulmonary and kidney failure. Endotoxin-related diseases are a leading cause of death among those patients in intensive care units.

Unique among antibiotics is the ability of polymyxins, especially of polymyxin B (PMB) to neutralize endotoxin, accomplished by binding to the lipid A region of the endotoxin molecule. Polymyxin B from Bacillus polymyxa (B. aerosporus) is a highly charged amphiphilic cyclic peptidolipid. It is also useful in combating various fungal infections, especially those arising in immunocompromised individuals.

However, PMB has some properties which renders it less than an ideal antibiotic. First, it has a short half-life in the body, requiring repeated dosages in order to be effective. Secondly, as it passes through the kidney it can cause extensive damage. Thirdly, at high doses it possesses neurotoxic properties which cause respiratory paralysis.

Previously, researchers have conjugated polymyxin to immobile or fixed molecules. See e.g. Issekutz, J. Immunol. Methods 61 (1983) 275-281, describing the binding of PMB to Sepharose. These conjugates, while useful in purification techniques, are not suitable for in vivo therapeutic use.

One approach to achieve pharmacological activity, increased duration, or decreased organ toxicity has involved the conjugation of drugs to large molecular weight macromolecules such as dextran, polyethylene glycol or polyvinylpyrrolidine. Attempts in this area of polymer conjugation have been met with only limited success, however. For example, the conjugated form of procainamide (an antiarrythmic drug) was less active and exhibited a shorter half-life than native procainamide (Schacht et al., Ann. NY Acad. Sci. (1985) 446 199-211). Similarly, a prostaglandin analog, B245, linked to a carrier, was less effective (by several log orders) than the native molecule [Bamford et al., Bioch. Biophys. Acta 886 (1986) 109-118]. Reductions in biological potency have also been described for conjugated forms of kallikrein, aprotinin, bradykinin [Odya et al., Biochem. Pharmacol. 27 (1978) 173-179] and the anti-tumor drugs daunorubicin [Hurwitz et al., J. Appl. Biochem. 2 (1980) 25-35] and mitomycin C [Takakura et al., Cancer Res. 44 (1984) 2505-2510]. Conjugated enzymes also suffer a reduction in biological activity due to steric hindrance and reduced substrate accessibility [Blomhoff et al., Biochem. Biophys. Acta 757 (1983) 202-208; Marshall et al., J. Biol. Chem. 251 (1976) 1081-1087; Foster, Experientia 31 (1975) 772-3; Wileman et al., J. Pharm. Pharmacol. 35 (1983) 762-765]. There are, however, some examples of improvements in circulatory half-life after conjugation [Wileman, supra; Kaneo, Chem. Pharm. Bull. 37 (1989) 218-220].

It would be desirable to develop a form of polymyxin which would stay in the blood stream longer and/or does not have neuro- or nephrotoxicity at therapeutic doses.

The present invention relates to polymyxin-carrier conjugates which are soluble in water, e.g. polymyin A-, B- or E-carrier conjugates, particularly PMB-carrier conjugates. They are indicated for use in neutralizing endotoxin. They are an improvement over the administration of native polymyxin because they are more effective and are less toxic.

As used herein the following definitions apply:

- LD_0 is the highest non-toxic dose of PMB or its conjugate;
- LD_{100} is the dose of PMB or its conjugated form which results in 90-100% lethality when injected into a normal test animal;
- PD_{100} is the dose of polymyxin B or its conjugated form which, when injected into a hypersensitized test animal, results in at least 95 % survival;
- Therapeutic Index (TI) is calculated by dividing LD_{100} by PD_{100}.

As defined herein a conjugate is water-soluble when it has a solubility in water of at least 0.5 mg/ml at 25° C, preferably of at least 1 mg/ml at 25° C. Such conjugates are water-soluble and injectable, non-matrix, colloid, not cross-linked, non-macromolecular, and/or non-particulate.

The carrier which can be conjugated to polymyxin is chosen from molecules which can form water-soluble conjugates and which are uniform, non-toxic, non-carcinogenic, non-irritating and non-immunogenic. It is normally a biopolymer. Such carriers include polysaccharides such as dextran or hydroxy ethyl starch (HES), proteins such as albumin, and polymers such as polyvinyl pyrrolidone, polyethylene glycol and polyvinyl alcohol. Dextran is the most preferred.

The size of the biopolymer portion, i.e. the carrier part of the conjugate may vary. Typically it will range from a molecular weight of 25,000 to 500,000, preferably from 50,000 to 300,000, more preferably from

79,000 to 200,000. The size of the biopolymer chosen can significantly contribute to the duration of the conjugate's effective time of circulation in the blood stream. Generally, the larger the biopolymer, the longer the conjugate will stay in the circulation. Thus the size of the biopolymer can be adjusted to result in a conjugate whose time of duration in the body corresponds to a predetermined time.

The conjugates of the invention may be prepared in conventional manner.

Taking polymyxin B as an illustration, this has five γ-amino groups which bind to the bacterial endotoxin. At least one γ-amino group can be used to securely bind the PMB to the carrier, but all five sites cannot be used for this purpose, or the conjugate will lack endotoxin-neutralizing activity. Various chemical reactions can be used to conjugate polymyxin to these carriers. The number of PMB molecules bound per molecule of polymer can be influenced by varying the ratios of reactants during the coupling reaction. Generally the conjugate will contain between 1 PMB : 15 biopolymer to 200 PMB : 1 biopolymer, more preferably the conjugate will have from 1-10 PMB : 1 biopolymer, and even more preferably from 1-3 PMB : 1 biopolymer on a molar basis. The conjugates with high PMB : biopolymer ratios seem to possess greater potency than conjugates with lower ratios.

One method of making a polymyxin-carrier conjugate, more specifically, a PMB-dextran conjugate, is through carbamate linkage (method A). PMB-dextran conjugates made in this fashion (detailed in Example 1, below) are found to retain the anti-endotoxin activity of native PMB, and in addition are found to be devoid of the acute neurotoxicity exhibited by native PMB. These conjugated forms also show a 2-5 fold improved therapeutic index by decreasing chronic toxicity over that of native PMB.

A second method of making conjugated polymyxin dextran is through an amine bond (method B). PMB-dextran conjugates made in this manner (detailed in Example 2, below) retain the same general anti-endotoxin activity or are more active than native PMB. These conjugated forms are completely devoid of any acute neurotoxicity seen by native PMB and exhibit a 33-fold improvement of the therapeutic index by reducing chronic toxicity over that seen with native PMB. In particular, one PMB-dextran conjugate is devoid of any measurable toxicity at the doses tested and retains good anti-endotoxin activity, resulting in an 80-fold improvement in the therapeutic index.

Further, it has been surprisingly found that conjugates produced by this method and "rapidly purified" exhibit a 60- to 120-fold improvement in TI. "Rapidly purified" as used throughout the specification and claims means that the material is purified from unbound PMB using molecular sieving chromatography, desalting gels, or Amicon ultrafiltration, occurs for approximately 12-24 hours, preferably approximately 18 hours (as opposed to an extended dialysis time of 7-10 days). Thus conjugates produced by these methods and rapidly purified comprise another and preferred aspect of this invention.

The polymyxin-carrier conjugate obtained from either of the two methods outlined above is, however, difficult to purify. Initially, a 10-day extended dialysis was tried in order to separate native PMB from the conjugate. Although the protein level inside the dialysis bag reached an asymptote, and gel permeation column chromatography showed a single large molecular weight species, the material still contained unbound PMB. Even when pressure dialysis (Amicon) filtration was substituted for extended dialysis the results were the same. Conjugates purified in this manner were equipotent to PMB, but still retained 1/3 to 1/5 the toxicity of PMB. While not intending to be bound by theory, it appears that there is some chemical association between the "free" PMB and the conjugate which renders its separation difficult. Upon injection in the animal the "free" PMB dissociates itself and causes toxicity problems.

The following procedure (method C) (detailed in Example 4, below) obviates the above difficulties. Polymyxin-dextran conjugates such as PMB-dextran conjugates are precipitated in a lower alcohol, preferably methanol, followed by centrifugal collection and ultrasonic resolubilization. This procedure is repeated, normally at least three times, and preferably 7 to 10 times and the resulting material is evaluated for purity, e.g. by gel permeation chromatography and reverse-phase high pressure liquid chromatography (RP-HPLC). If protein is detected by either method, the precipitation-resolubilization procedure is repeated. Conjugate purified in this manner is referred to herein as "ultra-pure" polymyxin-conjugate and generally can be described as having less than 20 μg unconjugated polymyxin per 1 mg total protein, and preferably less than 10 μg unconjugated polymyxin per 1 mg total protein.

A further aspect of the invention is thus a process for the preparation of a water-soluble polymyxin-dextran conjugate comprising

a) appropriately conjugating polymyxin to dextran to form a water-soluble polymyxin-dextran conjugate;

b) precipitating the resultant conjugate in a lower alcohol;

c) resuspending the conjugate; and

d) repeating steps b) and c) until the conjugate is substantially free from unbound polymyxin.

"Substantially free from unbound polymyxin" is herewith defined as a ratio of conjugate to unbound polymyxin of at least 95 : 1, preferably of at least 99 : 1 on a molar basis. The lower alcohol is e.g. of 1 to

10 carbon atoms, it preferably is of 1 to 4 carbon atoms, e.g. n-butanol or isopropanol, more preferably methanol or ethanol. The polymyxin preferably is PMB.

Ultra-pure conjugate has virtually no toxicity and as a result an LD_{100} cannot be established for it. At a dose of 100 mg/kg no toxicity is seen. At higher concentrations the solution is too viscous to inject. This is in contrast to native PMB which has an LD_0 i.v. of 5 mg/kg and an LD_{100} i.v. of 9.5 mg/kg. Thus the Therapeutic Index of ultra-pure PMB conjugate is over 1000-fold higher than native PMB, due to decreased toxicity.

A second advantage in using the conjugate rather than native polymyxin is that the conjugate has a longer duration of activity and thus can be used prophylactically. Thus one aspect of the invention is the prevention of diseases caused by the presence of bacterial endotoxin by administering to a subject in need of such prophylactic treatment a prophylactically-effective amount of a water-soluble conjugate of polymyxin, preferably PMB and a carrier, particularly dextran, and a pharmaceutically acceptable further carrier or diluent as appropriate, e.g. an inert solution. As the conjugate has virtually no toxicity it can be prophylactically administered on a routine basis to patients who might be susceptible to septic shock, such as those dependent upon liquid food for long-term nourishment.

The invention also concerns a pharmaceutical composition comprising a water-soluble polymyxin-carrier conjugate together with further pharmaceutically acceptable carriers or diluents as appropriate, e.g an inert solution.

The invention also concerns a process for the preparation of a pharmaceutical composition comprising mixing a water-soluble polymyxin-carrier conjugate with further pharmaceutically acceptable carriers or diluents as appropriate, e.g. an inert solution.

Another aspect of the invention is the treatment of diseases caused by the presence of bacterial endotoxin comprising administering to a subject in need of such treatment an endotoxin-neutralizing amount of a water-soluble conjugate of polymyxin, particularly PMB and a carrier, particularly dextran, and an inert solution. As polymyxin is also effective im combating bacterial or fungal infections, another aspect of this invention is a method of treating bacterial or fungal infections comprising administering to a subject in need of such treatment a bacteriocidally- or fungicidally-effective amount of a water-soluble conjugate of polymyxin and a carrier, particularly dextran, and an inert solution.

The polymyxin-carrier conjugate of the invention can be used in a manner consistent with the use of polymyxin itself, i.e. it can be used alone as an antibiotic for bacterial or fungal infections or combined with other bacteriocidal agents and/or anti-inflammatory agents. It may be administered, e.g. intramuscularly, intravenously, intrathecally, subconjunctivally or topically. Thus formulations for intramuscular injections typically comprise an effective amount of PMB-conjugate in sterile water, physiological saline or approximately 1 % procane•HCl. Intravenous formulations typically comprise an effective amount of PHB-conjugate in 5 % dextrose and sterile water. Intrathecal formulations typically comprise an effective amount of PMB-conjugate in physiologic saline. For topical ophthalmic use, an effective amount can be mixed with water or physiological saline, and optionally glycerine, and cupric sulfate for eye drops, or it may be made into an ointment or suspension. Creams, for topical applications, especially for burned areas, typically comprise an effective amount of polymyxin-conjugate in a base of inactive ingredients such as liquid petrolatum, propylene glycol, polyoxyethylene, polyoxypropylene and emulsifying wax.

The amount of polymyxin-conjugate to be used is based on the amount of native polymyxin which would typically be prescribed for a particular patient (taking into account such factors as the condition being treated and age and weight of the patient) and the activity of the particular polymyxin-conjugate used. Often, a dosage reduction of up to 50 % compared to native polymyxin can be realized due to the effective increased activity of the polymyxin conjugate, its reduced toxicity and its increased duration of activity.

The invention is illustrated by reference to the following non-limiting Examples. All temperatures are in degrees Centigrade.

Example 1: Chemical conjugation of PMB to dextran

(Method A; carbamate linkage)

2 g dextran (79,000 or 200,000 MW) is dissolved in 20 ml water, cooled to 0°, and 5-300 mg of 1-cyano-4-dimethylamino pyridinium tetrafluoroborate (CDAP) is added and mixed for 30 seconds. Triethylamine (0.2 M, 0.04 ml per 5 mg CDAP) is added dropwise with vigorous stirring, and the entire reaction mixture is transferred to 80 ml of ice cold absolute ethanol containing 1 ml of 10 N HCl. The

dextran precipitates, and the precipitate is removed by centrifugation at 1000 x g for 5 min at 0°, and resolubilized in 20-50 ml of 0.25 M Na-bicarbonate buffer at pH 9.0. To this mixture 600-1000 mg of PMB (either powdered or presolubilized in water) is added and stirred for 24 hours at 8°. The entire reaction mixture is then transferred to a 50,000 molecular weight cut-off dialysis tubing and dialyzed against 0.05 M pyrogen-free phosphate buffer for 6-10 days. The final dialyzed reaction mixture is measured for protein content by spectrophotometry at 208 nm absorbance or at 595 nm using the Bradford reagent (Bio Rad, Richmond, CA, USA).

Free amino groups are determined using the ninhydrin reaction, with native PMB as a control. Analysis for nitrogen and carbon content is done using a CHN Elemental Analyzer.

In the table below, "molar ratio" is determined by dividing the concentration of dextran used in conjugation (23.7 µmol) by the final PMB-protein concentration in µmol (based on 208 nm analysis) after dialysis. Dextran is abbreviated "dx". The "C:N" ratio assumes 450 molecules of water per 2 g dextran. The "bonds/PMB" is an estimate of the number of bonds by which PMB is attached to dextran, based on the ninhydrin reaction. "ND" is "not determined".

Summary of conjugation reactions				
Reaction	CDAP	molar ratio	C:N ratio	bond/PMB
A	21 µmol	1 dx : 0.23 PMB	ND	ND
B	106 µmol	1 dx : 1.62 PMB	1 dx : 1.52 PMB	3.0
BB	106 µmol	1 dx : 1.95 PMB	1 dx : 1.90 PMB	1.1
C	532 µmol	1 dx : 3.70 PMB	1 dx : 3.25 PMB	3.0
CC	532 µmol	1 dx : 4.99 PMB	1 dx : 5.00 PMB	2.2
D	1064 µmol	1 dx : 6.62 PMB	1 dx : 8.70 PMB	1.7
DD	1064 µmol	1 dx : 8.81 PMB	ND	1.3
E	2128 µmol	1 dx :10.9 PMB	ND	2.1

Example 2: Chemical conjugation of PMB to dextran

(Method B; amine bond)

1.25 g dextran (79,000 or 200,000 MW) is dissolved in 20 ml of distilled water and then 0.071-0.71 g Na-periodate is added. After 1 hour at 22° the reaction mixture is transferred to a column containing DEAE A25 cationic exchange resin (Pharmacia Inc., Piscataway, NJ, USA) and the mixture is collected and pooled to a single fraction of 20-25 ml. The oxidized dextran is then mixed with 2 g PMB dissolved in 80-200 ml Na-bicarbonate or Na-borate buffer (pH 8.5-9.0) and after 60 minutes, 40 ml 0.05 - 25 % Na-borohydride either in a single treatment or in multiple treatments, each treatment lasting 30 minutes to 24 hours is added. This reaction proceeds for 30 minutes and then is dialyzed for 7-10 days at 80 against 0.05 H pyrogen-free Na-phosphate buffer. The final dialyzed reaction mixture is analyzed for protein content and free amino groups as described in Example 1.

In addition to purification by extended dialysis, several representative samples are rapidly purified using dialysis for 18 hours, purified on a G-100 Sephadex column (Pharmacia, Inc., Piscataway, NJ, USA) and concentrated on an Amicon filtration unit using a YM-100 Amicon filter.

In the table below the molar ratio is determined by dividing the amount of dextran used in conjugation (6.25 µmol) by the µmol final PMB protein (based on the 208 nm data) after dialysis. The percent nitrogen is determined using a CHN elemental analyzer. Bonds/PMB is estimated by the ninhydrin reaction:

Summary of conjugation reactions				
Reaction	$NaIO_4$ (g)	molar ratio	% nitrogen	bonds/PMB
1/50	0.014	1 dx : 3.64 PMB	0.21	3.6
1/10	0.071	1 dx : 12.9 PMB	1.05	2.9
1/5	0.142	1 dx : 26.8 PMB	1.71	2.6
1/4	0.178	1 dx : 29.8 PMB	2.76	3.0
1/2[1]	0.355	1 dx : 39.6 PMB	3.74	2.2
stock*	0.710	1 dx :151 PMB	6.91	4.0

*Reaction mixture is turbid and remains so after dialysis
[1]Purified using rapid dialysis

Example 3: Anti-endotoxin activity of PMB-conjugates

A. Endotoxin-induced lethality

Male CB57BL/c mice (18-22 g, Jackson Labs) are used throughout this study. Animals are given endotoxin ($0111B_4$ from List Biologicals, Palo Alto, CA, USA) and galactosamine to hypersensitize, as described in Galanos et al., Proc. Natl. Acad. Sci. USA 76 (1979) 5939. The endotoxin and galactosamine (320 mg/kg) are injected intraperitoneally in 0.5 ml pyrogen-free isotonic saline per animal between 13:00 and 15:00 to avoid diurnal variation. From a dose-response study, it is determined that 0.01 mg/kg endotoxin produces 85-95 % lethality. Animals are observed each day for 6 days after injection.

B. PMB (native and conjugated forms) induced lethality

PMB (native and conjugated forms) are evaluated for their inherent toxicity in non-sensitized mice. Male CB57BL/c mice (18-22 g, Jackson Labs) are used throughout this study. Animals are given either native PMB or conjugated PMB by intraperitoneal injection (0.5 ml/animal) and lethality is monitored for 7 days. The LD_{100} is determined by varying the dose of PMB (native and conjugated forms) that result in 100 % lethality. From 10 to 20 animals are used for each dose.

C. PMB anti-endotoxin evaluations

PMB (native and conjugated forms) are evaluated for their ability to neutralize endotoxin by either pre-mixing with endotoxin for 60 minutes before i.p. injection or by coadministration of the substances as a single i.p. injection. While the concentration of the PMB (native and conjugated forms) are varied, the volume of each injection is kept constant at 0.5 ml per animal. The protection by the conjugated PMB is compared to the native PMB (in terms of mg protein/kg). Controls (endotoxin, galactosamine and vehicle) are included as well in each study. Each group has between 10 and 17 animals.

Statistical analyses of survival between groups or in relation to controls is performed using a Chi-square analysis using the Yate's correction for continuity.

D. Determination of Therapeutic Index

The Therapeutic Index (TI) for PMB (native and conjugated forms) is determined by dividing the LD_{100} by the PD_{100}. In the table below, the "treatment" refers to the conjugates appearing in Examples 1 and 2, supra.

Anti-endotoxin data			
Treatment	LD_{100} (mg/kg)	PD_{100} (mg/kg)	TI
Native PMB[1]	25	2	12.5
Native PMB[2]	25	2	12.5
A	>30	1	>30
B	>28	>2	>14
C	ND	5	ND
D	140	2	70
1/5*	83	0.2	415
1/2[1*]	about 40-60	0.05	800-1200
stock[2]	>212	0.2	>1060
stock[3]	>212	20	>10.6

[1]Rapidly-purified material
[2]The PMB or conjugate is allowed to react with the endotoxin for 60 min before i.p. injection
[3]The PMB or conjugate is injected simultaneously with the endotoxin
*The fractions designate reaction conditions based on the amount of sodium periodate used in the conjugation reaction. If 710 mg is multiplied by the fraction the amount (in mg) of sodium periodate used in each sample is obtained.

Example 4: Ultra-pure PMB-dextran conjugate

(Method C; alcohol precipitation)

1. Preparation

PMB-dextran conjugate is prepared essentially as described in Example 2, supra, to obtain conjugate designated "RXN 1/50".

The conjugate, a white flocculant, is precipitated in 60 % methanol, collected by centrifugation, and then subjected to ultrasonic resolubilization. This process is repeated seven times and the resulting material is evaluated for purity by gel permeation chromatography and reverse phase high pressure liquid chromatography (RP-HPLC). Although no free PMB is detected by gel permeation chromatography, RP-HPLC shows 48 µg unconjugated PMB per mg of total protein. The conjugate is then subjected to 3 additional precipitations to remove the unconjugated PMB.

2. Biological activity

The resulting ultra-pure PMB-dextran conjugate is compared to native PMB in an endotoxin-induced lethality test as described in Example 3, C., supra.

At 1 mg/kg i.v. the ultra-pure PHI-conjugate gives > 70 % protection against a LD_{90} endotoxin challenge for at least 6 hours. At 10 mg/kg i.v. this conjugate provides therapeutic protection 1.5 hours after administration of the endotoxin. Therefore the PMB-conjugate is suitable for prophylactic use.

The ultra-pure PMB-conjugate has a surprisingly reduced toxicity as compared with native PMB. Native PMB has an LD_0 i.v. of 5 mg/kg and an LD_{100} i.v. of 9.5 mg/kg, suggesting an early onset of toxicity and narrow therapeutic range. The ultra-pure PMB-conjugate has an LD_0 of > 100 mg/kg and it has not been possible to establish an LD_{100} due to its inherent non-toxicity. Comparisons in the table below are made between the highest non-toxic dose of PMB (native or conjugated):

Biological activity			
Treatment	LD_0 (mg/kg)[1]	PD_{100} (mg/kg)[2]	TI
PMB	5	0.1	50
ultra-pure conjugate	> 100	0.1	> 1000

[1] PMB (native or conjugate) given by i.v. injection and lethality monitored over 7 days

[2] PMB (native or conjugate) given by i.v. injection 1-3 minutes before an LD_{90} i.p. injection of E.coli 0111B4 endotoxin (0.5 µg/kg)

Claims

1. A water-soluble conjugate of a polymyxin and a carrier.
2. A conjugate according to claim 1 wherein the carrier is a polysaccharide such as dextran or hydroxyethyl starch, a protein such as albumin, or a polymer such as polyvinylpyrrolidone, polyethylene glycol or polyvinyl alcohol.
3. A conjugate according to claim 1 wherein the polymyxin is PMB.
4. A conjugate according to claim 2 wherein the carrier is dextran.
5. A conjugate according to claim 3 wherein the carrier is dextran.
6. A conjugate according to claim 5 wherein PMB is attached through carbamate linkage.
7. A conjugate according to claim 5 wherein PMB is attached through amine bonds.
8. A process for the preparation of a polymyxin-dextran conjugate according to claim 4 comprising:
   a) appropriately conjugating polymyxin to dextran to form a water-soluble polymyxin-dextran conjugate;
   b) precipitating the resultant conjugate in a lower alcohol;
   c) resuspending the conjugate; and
   d) repeating steps b) and c) until the conjugate is substantially free from unbound polymyxin.
9. A pharmaceutical composition comprising a conjugate according to claim 1 together with further pharmaceutically acceptable carriers or diluents.
10. A conjugate according to claim 1 for use as a pharmaceutical.

Claims for the following Contracting States:ES,GR

1. A process for the preparation of a pharmaceutical composition comprising mixing a water-soluble conjugate of a polymyxin and a carrier together with further pharmaceutically acceptable carriers or diluents.
2. A process according to claim 1 wherein the carrier is a polysaccharide such as dextran or hydroxyethyl starch, a protein such as albumin, or a polymer such as polyvinylpyrrolidone, polyethylene glycol or polyvinyl alcohol.
3. A process according to claim 1 wherein the polymyxin is PMB.
4. A process according to claim 2 wherein the carrier is dextran.
5. A process according to claim 3 wherein the carrier is dextran.
6. A process for the preparation of a water-soluble polymyxin-dextran conjugate comprising:
   a) appropriately conjugating polymyxin to dextran to form a water-soluble polymyxin-dextran conjugate;
   b) precipitating the resultant conjugate in a lower alcohol;
   c) resuspending the conjugate; and
   d) repeating steps b) and c) until the conjugate is substantially free from unbound polymyxin.
7. A process according to claim 6 wherein the conjugate is a PMB-dextran conjugate.
8. A process according to claim 6 wherein steps b) and c) are repeated at least three times.

European Patent Office

EUROPEAN SEARCH REPORT

Application Number

EP 90 81 0872

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. 5)
X	FR-A-2 342 740 (PHARMACO, INC.) * Page 3, line 6 - page 5, line 2; page 6, line 26; page 6, line 30 - page 7, line 9; page 11, line 31 - page 13, line 10; page 18, example 1; page 27, example IV; page 37, table IV * ---	1-10	A 61 K 47/48
X	FILE SERVER STN, Karlsruhe, FILE BIOSIS, abstract no. 85:421296, accession no. Biological abstract 80:91288; T. KITAGAWA et al.: "Sensitive enzyme immunoassay of colistin and its application to detect residual colistin in rainbow trout tissue", & J. ASSOC. OFF ANAL. CHEM. 68 (4), 1985, 661-664 * Abstract * ---	1-3	
Y	IDEM ---	1-10	
Y	EP-A-0 147 761 (MILES LABORATORIES INC.) * Page 1, lines 4-19; claims 1-8 * -----	1-10	TECHNICAL FIELDS SEARCHED (Int. Cl.5) A 61 K

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	08-02-1991	SITCH W.D.C.

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
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T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
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& : member of the same patent family, corresponding document